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Original Article

Bcl-2 as a Predictive Factor for Biochemical Recurrence after Radical Prostatectomy: An Interim Analysis

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Purpose
The objective of this study was to determine Bcl-2 expression in localized prostate cancer and its potential role as a predictive factor for biochemical recurrence (BCR).

Materials and Methods
This study included 171 Korean patients with newly diagnosed adenocarcinoma of the prostate who underwent radical prostatectomy (RP) without neoadjuvant therapy at a single center between February 2005 and May 2009. RP specimens obtained from these patients were analyzed for the expression of Bcl-2 using tissue microarray. The values of Bcl-2 and other clinicopathologic factors were evaluated. Statistical analysis was performed with contingency table analysis, chi-square tests, and a Cox proportional hazard model.

Results
Bcl-2 expression was immunohistologically-confirmed in 42 patients (24.6%). Bcl-2 expression was not associated with conventional clinicopathologic factors. Bcl-2 negative patients had a significantly longer mean BCR-free survival than Bcl-2-positive patients (p=0.036). Among several variables, a high Gleason score in the RP specimen ($\geq$8), extraprostatic extension, seminal vesicle invasion (SVI), lymphovascular invasion (LVI), and Bcl-2 expression were significant predictors of BCR based on univariate analysis. Multivariate Cox proportional hazards analysis revealed that BCR was significantly associated with a high prostate specific antigen level (p=0.047), SVI ($p < 0.001$), a positive surgical margin (p=0.004) and Bcl-2 expression (p=0.012).

Conclusion
Bcl-2 expression in RP specimens is associated with a significantly worse outcome, suggesting a potential clinical role for Bcl-2. Post-operative Bcl-2 could be a significant predictor of outcome after RP.

Key words
Prostatic neoplasms, Proto-oncogene proteins, B-cell leukemia/lymphoma 2, Recurrence, Prostatectomy

Introduction

The early detection of prostate cancer with prostate specific antigen (PSA) testing allows many patients the option of radical treatment with curative intent. However, approximately 25% of patients will develop a post-operative biochemical recurrence (BCR) (1). The prognosis after radical prostatectomy (RP) is usually based on clinical findings, such as the pre-operative PSA level and PSA doubling time, pathologic findings, such as the Gleason score, surgical margin status (SMS), extraprostatic extension, and seminal vesicle invasion (SVI) (1-5). Furthermore, several investigators have reported the usefulness of nomograms in calculating survival after definitive therapy for localized prostate cancer, which were developed based on multiple clinical parameters. To date, numerous attempts have been made to use tissue biomarkers to enhance the prediction of outcome after RP. However, no molecular markers have been identified that contribute independent prognostic information on prostate specimens to enable prediction of treatment failure after RP. Many potentially prognostic markers have been studied, and some have been incorporated into prognostic models or therapeutic decision making. The oncoprotein encoded by the Bcl-2 gene is well-accepted

for its involvement in the development of human follicular B-cell lymphomas (6). Likewise, this gene is suspected of participating in the development of other types of human malignancies (lymphomas, and breast and prostate cancer) (7-9). Unlike in breast cancer and lymphoma, a report by Berges et al. (10) suggests that p53 gene expression is not required to mediate programmed cell death in androgen-deprived prostatic glandular epithelial cells in prostate cancer. Furthermore in clinical specimens, it is not clear that p53 and bcl-2 necessarily interact in apoptosis mechanisms. Bauer et al. (11) reported that only 3 of 40 (7.5 percent) adenocarcinoma specimens of the prostate exhibited combined p53 and bcl-2 positivity. Bcl-2 overexpression is a relatively low-frequency event in clinically localized prostate cancer. In addition, bcl-2 showed higher specificity and positive predictive value than that of p53. Thus, in prostate cancer, we think that bcl-2 could be an independent biomarker.

In this study, we analyzed the expression of Bcl-2 in a group of Korean patients to ascertain the clinical significance of Bcl-2 expression in relation to BCR after RP.

Materials and Methods

1 Study population

One hundred and seventy-one Korean men, 49-80 years of age, with clinically localized adenocarcinoma of the prostate were included in this study. The patients underwent RP at the Korean National Cancer Center (NCC) between February 2005 and May 2009. None of the patients received androgen deprivation therapy before RP. All patients had a pre-treatment serum PSA, which ranged from 0.8 to 79 ng/mL (mean, 9.8 ng/mL). TNM (primary tumor, regional nodes, metastasis) classification was performed according to the 2002 American Joint Committee on Cancer. The margin was considered positive when cancer cells were detected in ≥ 1 surgical margins.

In accordance with the requirements of our institutional review board, informed consent to the prospective study was obtained from each patient, and the study design was approved by the Research Ethics Committee of our institution. The mean duration of follow-up after RP was 23.3 months (range, 2 to 51 months). After RP, patients were followed up by measurements of serum PSA levels at ≤ 3 month intervals for the first 2 years and every 6 months thereafter. A BCR was defined as a post-operative serum PSA of at least 0.4 ng/mL. Irrespective of pathologic findings, suggesting a poor prognosis, none of the patients received any adjuvant therapy until BCR was detected.

2 Immunohistochemical technique

RP specimens obtained from these patients were analyzed for the expression of Bcl-2 using tissue microarray (TMA). Paraffin-embedded formalin-fixed prostatectomy tissue blocks were cut in 3 μm thicknesses onto silane-coated slides and heated for 30 minutes in an 57℃ oven. The tissue was then deparaffinized in xylene, rinsed well over several ethanol washes (95%), and finally with distilled water. The slides were then microwaved in EDTA buffer (pH 8.0) for 60 minutes for antigen retrieval. Following a rinse with water, 3% hydrogen peroxide was applied to the slides for 4 minutes at room temperature.

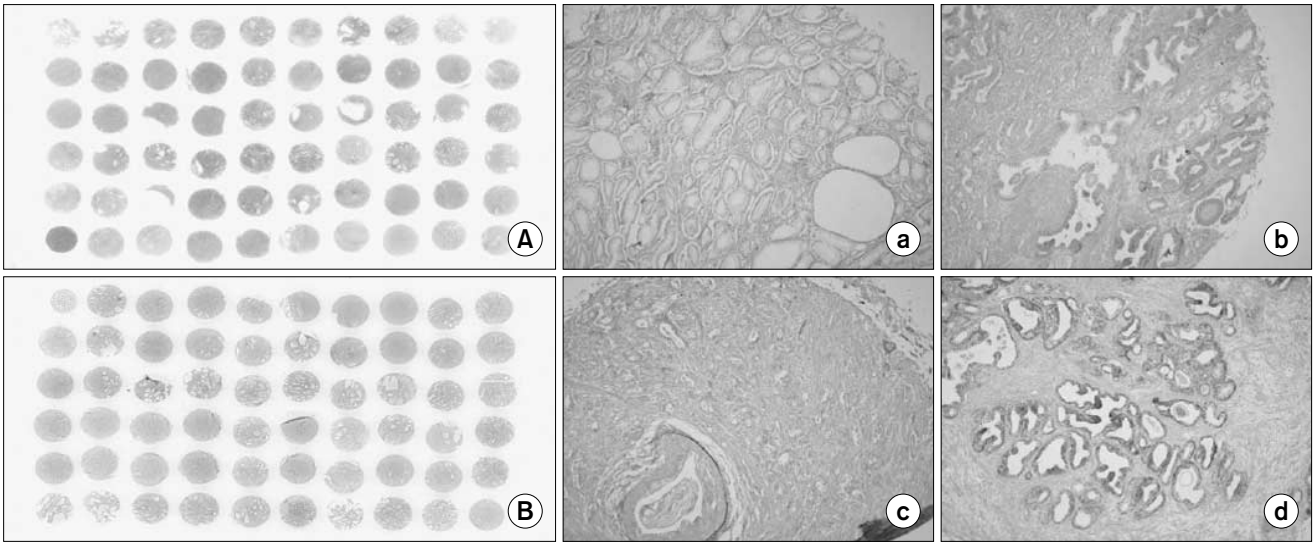

Fig. 1. Prostate prognostic tissue microarray (TMA) containing specimens from 171 tumors. (A) TMA section stained with hematoxylin and eosin (×100). (B) TMA section with Bcl-2 staining (×100). (a) Intensity of immunostaining for Bcl-2: negative group (×400). (b) Intensity of immunostaining for Bcl-2: weak group (×400). (c) Intensity of immunostaining for Bcl-2: moderate group (×400). (d) Intensity of immunostaining for Bcl-2: strong group (×400).

Another rinse with Tris buffer was performed before the primary Bcl-2 antibody (Dako co., Carpinteria, CA) was overlaid for 32 minutes at 42℃. The slides were again rinsed in Tris buffer twice, then incubated with Biotin (iVIEW DAB detection kit, Ventana, Tucson, AZ) for 10 minutes, rinsed again as before, and incubated with streptavidin for 8 minutes. After a final rinse with Tris buffer, chromogen (dimethylaminoazobenzene, Ventana) was applied for 8 minutes, followed by copper solution for 4 minutes. Counterstaining was then performed with commercially prepared hematoxylin for 4 minutes. After post-counterstaining was performed with bluing solution, the slides were dehydrated and coverslipped with Permount. BenchMark XT (Ventana) was used for all staining. Two independent uropathologists (WSP and EKH) viewed and interpreted the stained slides without knowledge of patient outcome. As shown in Fig. 1, the intensity of immunostaining for Bcl-2 was visually scored, and stratified into four groups (negative, weak, moderate, and strong); each group was defined as having no tumor cells, < 10% the tumor cells, 10% to 50% tumor cells, and > 50% tumor cells, respectively. Any cytoplasmic staining with Bcl-2 was considered positive, indicating expression. Staining in the basal epithelial cells of normal prostate epithelium served as an internal control.

3 End points and statistical analysis

Because there were too few patients with Bcl-2 weak, moderate, and strong staining, it was necessary to combine the groups which stained weak, moderate, and strong for this analysis. Contingency table analysis and chi-square tests were used to determine the relationship between the expression of Bcl-2 and other clinicopathologic features. In addition, we determined the impact of Bcl-2 on BCR. Recurrence-free survival curves were plotted according to the Kaplan-Meier method. A log-rank test was applied to determine the relationship between prognostic markers and BCR. A Cox proportional hazard model with stepwise selection of the co-variates was used to determine the parameters with the greatest influence on the risk of BCR. The level of statistical significance was set at $p < 0.05$ (two-sided), and the SPSS ver. 12.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis.

Results

1 Patients

Data on all 171 patients were available. The mean age was 64.4 years (49 to 80 years). The patient characteristics are listed in Table 1. The pre-treatment serum PSA 4～10 group was most common in our study. Pre- and post-operative Gleason scores ranged from 5 to 10. One hundred and sixty-seven patients had pre- and post-operative Gleason scores. The post-operative Gleason scores were higher than the pre-operative scores ($p < 0.001$). SVI, perineural invasion (PNI), and lymphovascular invasion (LVI) were detected in 13 (7.8%), 104 (61.2%), and 16 (9.4%) patients, respectively. The surgical margin positive rate was 34.7%.

Table 1. Patient characteristics

Classification	No. of patients (%)
PSA* (ng/mL)	
≤4.0	13 (7.6)
4.1-10.0	108 (63.2)
10.1-20.0	36 (21.1)
≥20.1	14 (8.2)
Pre-operative GS†	
5-7	159 (93.0)
8-10	12 (7.0)
Post-operative GS	
5-7	153 (91.6)
8-10	14 (8.4)
Clinical T stage	
cT1	88 (51.5)
cT2	74 (43.3)
cT3	9 (5.3)
cT4	0 (0.0)
Pathologic T stage	
pT2	126 (73.7)
pT3	45 (26.3)
pT4	0 (0.0)
Seminal vesicle invasion	
Present	13 (7.8)
Absent	154 (92.2)
Perineural invasion	
Present	104 (61.2)
Absent	66 (38.8)
Lymphovascular invasion	
Present	16 (9.4)
Absent	151 (88.3)
Surgical margin status	
Positive	58 (34.7)
Negative	109 (65.3)
Bcl-2	
Positive	42 (24.6)
Negative	129 (75.4)

*prostate-specific antigen, †gleason score.

2 Bcl-2 expression and clinicopathologic factors

Bcl-2 expression was immunohistologically confirmed in 42 patients (24.6%). The association between several clinicopathologic factors and Bcl-2 expression in RP specimens was analyzed. Bcl-2 staining was not associated with PSA level, pre-operative Gleason score, post-operative Gleason score, clinical stage, SVI, LVI and SMS.

3 The impact of Bcl-2 on BCR

During the present observation period, BCR developed in 15 of 171 patients (8.8%); there were BCRs in 6 of 41 patients (14.6%) with Bcl-2-positive RP specimens and in 9 of 126 patients (7.1%) with Bcl-2-negative RP specimens. The Kaplan-Meier survival estimates by Bcl-2 status are shown in Fig. 2. BCR-free survival in patients with Bcl-2 positive specimens was significantly lower than in patients with Bcl-2 negative specimens (p=0.036). At the time of mean follow-up, 23.3 months, BCR-free survivals of Bcl-2 positive and negative patients were 87.8% and 94.4% respectively. However, these findings were not significant statistically. Furthermore, we evaluated the predictive value of several clinicopathologic factors for BCR. Eight variables recorded at the time of prostatectomy were considered potential prognostic factors for BCR. By univariate Cox proportional hazards analysis, the post-operative Gleason score ≥8 (p=0.002), extraprostatic extension (p=0.006), SVI (p<0.001), LVI (p=0.008), and Bcl-2 expression (p=0.039) significantly influenced the time to BCR. Furthermore, the pre-operative Gleason score (p=0.060) influenced BCR with borderline significance. Multivariate Cox proportional hazards analysis revealed that BCR was significantly associated with a high PSA level (hazard ratio [HR], 10.147; p=0.047), SVI (HR, 42.613; p<0.001), a positive surgical margin (HR, 6.294; p=0.004), and Bcl-2 expression (HR, 4.503; p=0.012) (Table 2).

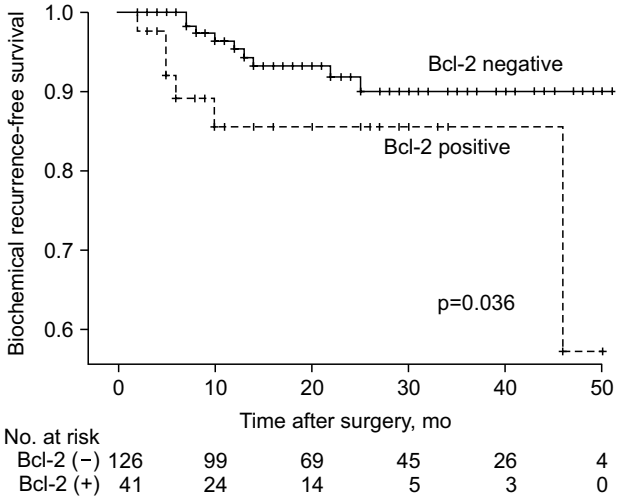

Fig. 2. Kaplan-Meier biochemical recurrence (BCR)-free survival curves according to the expression of Bcl-2.

Discussion

Carcinoma of the prostate varies greatly in biological behavior, ranging from slowly progressive to highly aggressive metastasizing tumors. The advent of immunohistochemical staining has led to many studies using tumor biomarkers, but no marker has moved from the research setting into the routine assessment of prostate carcinoma. Several groups have studied Bcl-2 with conflicting results (12). Given the alleged role of Bcl-2 expression for hormone resistance in prostate cancer, such a result would be expected after anti-androgen treatment (13-15). However, none of our patients had undergone neoadjuvant or adjuvant anti-androgen therapy, thus our results cannot be explained by this mechanism. Bauer et al. (11) reported that increased expression of Bcl-2 correlates with higher rates of BCR in patients who have undergone a RP for clinically-localized prostate cancer. Further studies have also shown the ability of Bcl-2 to be of prognostic value for patients with organ-confined disease (16,17). However, other studies have shown that Bcl-2 does not have prognostic significance in prostate cancer patients (18).

In the current study, the routine clinical outcome predictors (pre-operative PSA level, biopsy Gleason score, Gleason score in the RP specimen, pathologic stage, SVI, PNI, LVI, SMS) and the expression of Bcl-2 in specimens from 171 RPs performed during a 5-year

Table 2. Univariate and multivariate analyses of prognostic factors for biochemical recurrence

Variables	Univariate		Multivariate	
	HR* (95% CI†)	p-value	HR (95% CI)	p-value
Conventional				
PSA‡ (≤20.0 vs. ≥20.1§)	1.691 (0.220-12.990)	0.613	10.147 (1.032-99.781)	0.047
Pre-operative GS‖ (5-7 vs. 8-10§)	3.369 (0.949-11.964)	0.060	2.459 (0.473-12.773)	0.284
Post-operative GS (5-7 vs. 8-10§)	5.490 (1.862-16.184)	0.002	1.531 (0.325-7.202)	0.590
Extraprostatic extension (absence vs. presence§)	4.257 (1.512-11.984)	0.006	1.133 (0.258-4.963)	0.869
Seminal vesicle invasion (absence vs. presence§)	18.571 (6.565-52.531)	<0.001	42.613 (8.120-223.631)	<0.001
Lymphovascular invasion (absence vs. presence§)	4.343 (1.470-12.829)	0.008	2.683 (0.695-10.353)	0.152
Surgical margin status (negative vs. positive§)	2.012 (0.720-5.620)	0.182	6.294 (1.795-22.066)	0.004
Biomarker				
Bcl-2 expression (negative vs. positive§)	2.712 (1.162-7.646)	0.039	4.503 (1.384-14.651)	0.012

*hazard ratio, †confidence interval, ‡prostate-specific antigen, §reference category, ‖gleason score.

period were compared. Based on the contingency table analysis and chi-square tests, we did not find a significant relationship between Bcl-2 expression and other parameters. Several previous studies showed that Bcl-2 expression was not significantly associated with Gleason score or stage. For example, in a report of patients with Stage B, C, or D disease, Amirghofran et al. (19) found that Bcl-2 expression had no relationship to prostate carcinoma stage or Gleason score.

Our analysis of conventional clinicopathologic parameters showed that the post-operative Gleason score, extraprostatic extension, SVI, and LVI were predictors of BCR by univariate analysis and that a high PSA level, SVI, and positive surgical margin were significant by multivariate analysis. A notable finding was that the expression of Bcl-2 was an independent prognostic factor in univariate and multivariate analyses. Furthermore, based on the Kaplan-Meier curve, Bcl-2 expression was associated with a shortened BCR-free survival in our series.

Several groups have studied Bcl-2, p53, and Ki-67 immunostaining, but the results have been variable (11,16,20-22). The reasons for the differences in these results include various definitions of BCR, differences in the sensitivity of the commercial antibodies, and carcinogenetic differences in marker expression. Marks et al. (23) reported that prostate cancer specimens from native Japanese and Japanese-American men were histologically-similar, but tissue biomarker expression suggested differing mechanisms of carcinogenesis. In our study, study populations were native Korean men. We concluded that racial, ethnicity, and environmental differences affect expression of biomarkers. Based on the results reported herein, Bcl-2 appears to be an independent prognostic factor for BCR. This result implies that Bcl-2 expression in a Korean population has unique clinical significance.

A number of factors may have contributed to the differences between the findings of the current study and earlier studies. First, this study was a planned prospective study. Second, unlike the previous studies, the current study used a well-defined and relatively homogeneous group of patients. In a number of the earlier studies, heterogeneous groups of patients with pathologic stages ranging from pT1-pT4 were used, with some studies including patients with anti-androgen therapy or multiple ethnic groups. As a result, substantial variation in the risk of BCR would have related to tumor stage and Gleason grade, and these biologic differences would have contributed to potentially non-linear changes in biomarker expression that may not have extrapolated to clinical subgroups. Third, in contrast to earlier studies, two uropathologists (WSP and EKH) viewed the stained slides without knowledge of patient outcome in our study. This fact strengthens the significance of our study. In similarity to previous studies, the total Bcl-2 positive rate of our study group was 24.6% (11). In contrast to previous studies, on the other hand, the weak positive rate for our study group was 17.5%. We, thus, feel that pathologist subjectivity may have affected the results of previous studies.

We would like to address several limitations of this study. First, a sample size of 171 patients in as common a disease as prostate cancer is not sufficiently large. Second, the average duration of follow-up of 23.3 months was relatively short. This reduces the clinical significance of our relatively low BCR rate. Third, it is possible that the high rate of positive surgical margins and the low BCR rate may have influenced the statistical analyses. Furthermore, although this study focused on patients who were diagnosed with organ-confined prostate cancer, the clinicopathologic features were relatively adverse compared with the large series performed in Western country (24). Nevertheless, the research focusing on Bcl-2 and other molecular markers is worthwhile. Therefore, in the future, studies with larger numbers of patients and longer-term follow-up will be required. Fourth, the TMA has some limitations compared with real-time PCR. The most important potential limitation is the small size of the tissue sample (tissue volume). Thus, strong heterogeneity of the tumor sample may not be reflective of the heterogeneity of the tumor as a whole (18).

Conclusion

The expression of Bcl-2 in RP specimens seems to be associated with a significantly worse outcome, highlighting a potential clinical role. Post-operative Bcl-2 could be a significant predictor of BCR in patients undergoing RP. In addition, Bcl-2 expression may influence the decision for adjuvant or salvage treatment.

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